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Unwinding associated with synapsis of DNA molecules by recA protein

(genetic recombination/recA synaptic structure/homologous pairing/joint molecules/helicase)

ANNA M. WU, MARCO BIANCHI, CHANCHAL DASGUPTA, AND CHARLES M. RADDING

Departments of Human Genetics and Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **In the presence of adenosine 5'-[γ-thio]triphosphate, a nonhydrolyzable analog of ATP, *Escherichia coli* recA protein extensively unwinds duplex DNA in a reaction that is strongly stimulated by either homologous or heterologous single-stranded DNA [Cunningham, R. P., Shibata, T., DasGupta, C. & Radding, C. M. (1979) *Nature (London)* 281, 191–195]. In the presence of ATP and homologous circular single-stranded DNA, recA protein also unwinds circular duplex DNA that is nicked at a heterologous site. When DNA ligase seals this nick, the product is a highly negatively superhelical molecule that can be relaxed by *E. coli* topoisomerase I. This unwinding requires a high degree of homology since φX174 single-stranded DNA does not serve as a cofactor in the unwinding of G4 DNA, even though these molecules are 70% homologous. Like synapsis itself, and unlike strand exchange which follows synapsis, unwinding is sensitive to inhibition by ADP. Because recA protein unwinds duplex DNA when neither the single-stranded DNA nor the duplex DNA has a free end in the region of homology, unwinding can be initiated or mediated by a synaptic structure that differs from that of a simple D loop. The paired circular single strand in the synaptic structure behaves like one strand of an under-wound helix because *E. coli* topoisomerase I can interwind it with its complement.**

Purified *Escherichia coli* recA protein pairs duplex DNA with homologous single-stranded or partially single-stranded DNA; the product of the reaction is a joint molecule in which the two parental contributions are linked only by the hydrogen bonds of complementary bases. recA protein promotes not only the initial recognition or synapsis of two molecules but also an exchange of strands that can produce long heteroduplex joints (for reviews, see refs. 1–3).

Three phases can be distinguished experimentally in the overall pairing reaction: (*i*) a presynaptic phase during which recA protein polymerizes on single-stranded DNA (4–6), (*ii*) synapsis, which produces a nascent heteroduplex joint (7–9), and (*iii*) strand exchange, a polarized process that can either lengthen heteroduplex joints or cause their dissociation, depending on the substrates involved (9–14).

The energy that drives this sequence of reactions comes at least in part from ATP. The energy associated with binding of ATP appears to play an important role in the binding of single-stranded DNA, which in turn promotes the binding of duplex DNA (15). The hydrolysis of ATP is required for unidirectional strand exchange (8, 14).

In the presence of adenosine 5'-[γ-thio]triphosphate (ATPγS), single-stranded DNA binds tightly to recA protein and stimulates the binding of duplex DNA. All of the duplex DNA bound under these conditions is extensively unwound. The single-stranded DNA that stimulates unwinding in the presence of ATPγS need not be homologous to the duplex DNA (16, 17). The unwinding performed by recA protein in the presence of ATPγS has been related to a structure containing 18.6 base pairs and 6.4 molecules of recA protein per turn of a highly regular nucleoprotein fiber (18, 19).

The use of the analog ATPγS in studies of recA protein has allowed the ready observation of partial reactions that plausibly are steps in the overall formation of joint molecules. Ideally, however, one would like to check inferences drawn from the use of an analog by studies of normal reactions in the presence of ATP. We have found that single-stranded DNA stimulates the binding of duplex DNA in the presence of ATP (ref. 20 and unpublished data). Indirect evidence has also suggested that synapsis in the presence of ATP is mediated by a nucleoprotein structure in which recA protein holds the strands of the duplex molecule in an unwound conformation (9).

The experiments described in this paper were undertaken to look for direct evidence of unwinding of duplex DNA by recA protein in the presence of ATP. More specifically, we sought to study unwinding that might precede or accompany synapsis rather than the unwinding that necessarily accompanies subsequent strand exchange. While these studies were in progress, Ohtani *et al.* (21) kindly communicated to us their parallel observations on the unwinding of superhelical DNA by recA protein in the presence of ATP and homologous single-stranded DNA.

METHODS

Enzymes and DNA. recA protein was purified as described (22). Restriction endonucleases *Xho* I and *Bam*HI were purchased from New England BioLabs. Creatine phosphokinase (type I) was from Sigma. *E. coli* topoisomerase I was a gift of J. Wang.

Circular single-stranded and circular duplex DNA from phages G4, φX174, M13, and M13G*ori*1 were prepared as described (7, 23, 24). The preparations of single-stranded circular DNA contained <5% linear molecules as judged by electrophoresis in 1.8% agarose gels. All form I DNA (superhelical DNA) was purified by banding in CsCl/ethidium bromide gradients before site-specific nicking.

G4 or M13G*ori*1 form I DNA was specifically nicked by *Xho* I in the presence of ethidium bromide as described (25). Different preparations contained 70–85% form II (nicked circular duplex DNA), 15–30% form III (linear duplex DNA), and occasionally 1–2% form I (superhelical DNA). M13G*ori*1 DNA was nicked by *Bam*HI by digestion of form I DNA (150 μM) in 20 mM Tris·HCl, pH 7.6/100 mM NaCl/7 mM $MgCl_2$/2 mM 2-mercaptoethanol containing ethidium bromide at 75 μg/ml and *Bam*HI at 300 units/ml for 2 hr at 37°C. The preparation contained 30% form II and 70% form III DNA. Concentrations of DNA are expressed in moles of nucleotide residues.

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Abbreviation: ATPγS, adenosine 5'-[γ-thio]triphosphate.

Agarose Gel Assay for Unwinding. The standard recA reaction mixture (40 μl) contained 31 mM Tris·HCl (pH 7.6), 12 mM $MgCl_2$, 1.2 mM ATP, 100 μg of bovine serum albumin per ml, 24 μM nicked circular duplex DNA, 8–12 μM single-stranded circular DNA, 1.8 mM dithiothreitol, 2% (vol/vol) glycerol, and 4 μM recA protein. ATPγS (0.5 mM, Boehringer Mannheim) replaced ATP where indicated. The ATP-regenerating system consisted of creatine phosphokinase at 5 units/ml and 1.5 mM phosphocreatine unless otherwise indicated. After incubation at 37°C, $(NH_4)_2SO_4$ was added to 10 mM and NAD (Sigma), to 25 μM. One unit of *E. coli* DNA ligase (New England BioLabs) was added and incubation was continued at 37°C for 15 min. Samples were deproteinized by bringing the reaction mixture to 40 mM in EDTA and 0.1% in $NaDodSO_4$ and incubating with proteinase K (150 μg/ml; Miles) for 15 min at 37°C.

Vertical agarose gels contained 1.0% agarose (SeaKem LE) in 40 mM Tris acetate, pH 8.0/5 mM Mg acetate/0.5 mM EDTA. Electrophoresis was at 3 V/cm for 16 hr at 4°C with recirculation of the buffer. Magnesium was removed by rinsing extensively with distilled H_2O before staining the gel in ethidium bromide (1 μg/ml). Bands were assayed for radioactivity after dissolution of gel slices in a total volume of 0.6 ml of 1 M HCl at 95°C for 5 min and addition of 5 ml of Liquiscint (National Diagnostics, Somerville, NJ).

Relaxation of Superhelical DNA by Topoisomerase I. In order to show that the products of the ligation reaction were negatively superhelical, we treated samples with *E. coli* topoisomerase I. recA protein and ligase were inactivated by heating the reaction mixtures to 65°C for 5 min. *E. coli* topoisomerase I was added to a final concentration of 30 μg/ml, and incubation was at 37°C for 40 min. NaCl was added to a final concentration of 1 M, and incubation was continued at 37°C for 5 min to allow the topoisomerase to dissociate from the DNA.

Isopycnic Centrifugation in CsCl/Propidium Diiodide. Samples contained 500 μg of propidium diiodide (Sigma) per ml, 10 mM Tris·HCl (pH 7.6), 1 mM EDTA, and sufficient CsCl to make the density 1.565 g/cm^3. Centrifugation was in a Beckman SW 50.1 rotor at 30,000 rpm for 40 hr at 15°C. Gradients were collected from the bottom of the tube and assayed in Liquiscint (National Diagnostics).

RESULTS

Experimental Design. Experimentally, one can isolate an early phase of homologous pairing by using circular single-stranded DNA and a duplex molecule in which the homologous region is flanked on both sides by heterologous DNA. In the absence of a free end, the circular single strand cannot extensively interwind with its complement to form a right-handed helical heteroduplex joint. Nonetheless, from these substrates, recA protein efficiently forms joint molecules that are less stable than those containing classical heteroduplex joints (7, 24). Adopting terms coined some years ago (26), we shall call the structure formed by noninterwound complementary strands a "paranemic joint" and the classical interwound form of complementary strands, a "plectonemic joint."

For the experiments described here, we used M13G*ori*1 duplex DNA (27) which contains 2,216 pairs of G4 DNA inserted in 6,407 base pairs of M13 DNA (Fig. 1). By nicking this DNA in either the M13 region or the G4 region and using circular single-stranded DNA homologous to the other region, we could observe the effects of pairing in a region of "buried" homology. The effect of such pairing on unwinding the duplex molecule was determined by sealing the nick in the heterologous region with DNA ligase.

Because we previously observed that ADP, which accumulates during the pairing reaction, inhibits the initial formation of joint molecules (9), we did all of the experiments described here in the presence of an ATP-regenerating system, except as noted.

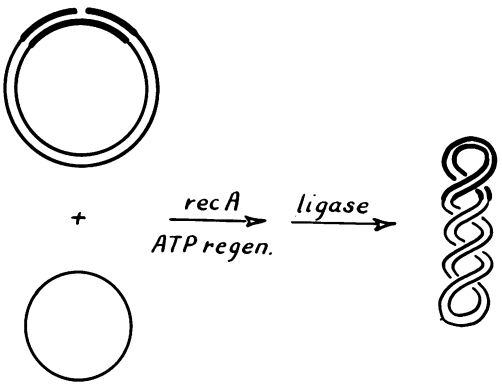


FIG. 1. Substrates and experimental design. The duplex DNA was M13G*ori*1 which contained 2,216 base pairs of G4 DNA (thick lines) inserted into 6,407 base pairs of M13 DNA (thin lines). A nick is shown here at the *Xho* I site in G4 DNA. A single *Bam*HI site exists near the middle of the M13 region. M13G*ori*1 DNA nicked in the G4 region by *Xho* I was allowed to react with single-stranded M13 DNA. Alternatively, M13G*ori* DNA was nicked in the M13 region by *Bam*HI and allowed to react with single-stranded G4 DNA (see Table 1). Changes in the conformation of the duplex DNA due to homologous interaction with single-stranded circular DNA were measured after sealing the nick in the heterologous region with DNA ligase.

Unwinding of Duplex DNA by recA Protein in the Presence of ATP and Single-Stranded DNA. When M13G*ori*1 DNA, nicked in the G4 region, was incubated with recA protein plus circular single-stranded M13 DNA and subsequently treated with *E. coli* DNA ligase, two new components were detectable by agarose gel electrophoresis (Fig. 2): one new band migrated at the position of form I M13G*ori*1 DNA, and the other, which was more diffuse, migrated faster (band X). In the samples that contained no single-stranded DNA there was no detectable form I band or X band (Fig. 2, lane 1).

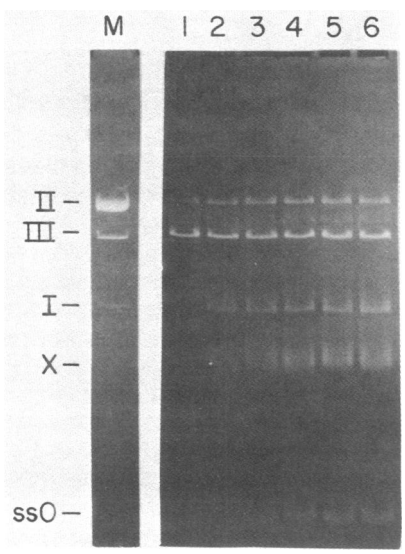


FIG. 2. Unwinding of duplex DNA by recA protein in the presence of ATP and single-stranded DNA. Lane M contained M13G*ori*1 DNA markers: form II (nicked circular duplex DNA), form III (linear duplex DNA), and form I (negatively superhelical DNA). Lanes 1–6 were from standard reaction mixtures containing 50 units of creatine phosphokinase per ml, and 2.3 mM phosphocreatine, and single-stranded circular M13 DNA (ssO) at 0 μM, 4 μM, 6 μM, 8 μM, 10 μM, and 12 μM. X, highly negatively superhelical DNA produced in the reaction (see text). Incubation was for 20 min at 37°C. In lane 1, relaxed closed circular DNA formed a set of bands that did not reproduce photographically; examples of such DNA can be seen in Figs. 3 and 5.

In another experiment, we cut the gel into slices and measured the radioactivity in the various bands (Table 1). In the presence or absence of recA protein, DNA ligase sealed two-thirds of the form II DNA. In the presence of recA protein and single-stranded DNA, 28% (in one experiment) and 57% (in another) of the ligated forms of DNA appeared in the form I band and the X band. The form I band and X band appeared either when the nick was in the G4 region of M13G*ori*1 and the single-stranded DNA was M13 or when the nick was in the M13 region and the single-stranded DNA was G4.

Characterization of the Products of the Reaction. In an experiment in which the single-stranded DNA was labeled with ^{32}P and the duplex DNA was labeled with ^{3}H, no ^{32}P was detected in either the form I band or in the X band. Thus, the two new bands detected by gel electrophoresis contained only duplex DNA.

We postulated that the DNA in the form I band was a form I-like component and that the X band contained more highly negatively superhelical DNA. Form V DNA, the limiting case of negatively superhelical DNA, with a linking number of 0, migrates in a similar way (28). To test this hypothesis, we treated the products of the reaction with *E. coli* topoisomerase I (ω protein) which relaxes only negatively superhelical DNA (29, 30). Topoisomerase I relaxed all of the DNA in the form I band and the X band (Fig. 3).

We assessed the extent of unwinding by examining the product of the reaction in a gradient of CsCl plus propidium diiodide (Fig. 4). The unwound molecules were heterogeneous; there was much material in the portion of the gradient corresponding to form I DNA and even more material corresponding to DNA that had been unwound 3–4 times as much as form I DNA (Fig. 4*C*). This highly unwound material presumably corresponds to the material that gives rise to the X band on gel electrophoresis. The CsCl/propidium diiodide gradient further shows that the topologically changed DNA is negatively superhelical.

Table 1. Unwinding of chimeric M13-G4 DNA promoted by either M13 or G4 single-stranded DNA

	% of total ^{3}H cpm in each lane					
	M13 single-stranded DNA			G4 single-stranded DNA		
		After ligation			After ligation	
	Start	No recA	With recA	Start	No recA	With recA
Unligated forms:						
Form II	85	30	29	29	10	9
Form III	14	14	14	70	70	70
Total	99	44	43	99	80	79
Ligated forms:						
Relaxed		55	41		18	9
Form I	1	1	9	1	1	8
X band		0	7		1	4
Total	1	56	57	1	20	21

Reaction mixtures contained either 24 μM M13 G*ori*1 [^{3}H]DNA nicked by *Xho* I and 8 μM M13 single-stranded circular DNA or 24 μM M13G*ori*1 [^{3}H]DNA nicked by *Bam*HI and 8 μM G4 single-stranded circular DNA (see Fig. 1). The ATP-regenerating system was included, and incubation was for 25 min at 37°C. After electrophoresis on an agarose gel and staining with ethidium bromide, we cut out appropriate regions of the gel, dissolved them by heating in 0.6 ml of 0.1 M HCl for 5 min at 95°C, and measured the radioactivity in 5 ml of Liquiscint fluor (National Diagnostics). The relaxed closed forms consisted of a ladder of bands containing the set of topoisomers that results from sealing relaxed nicked circular DNA (see Fig. 5 for clear example). Because the ladder of closed forms overlapped the form III position and the percentage of form III presumably remains constant from lane to lane, we cut out and assayed the entire ladder region and subtracted the contribution made by form III DNA in the nonligated controls.

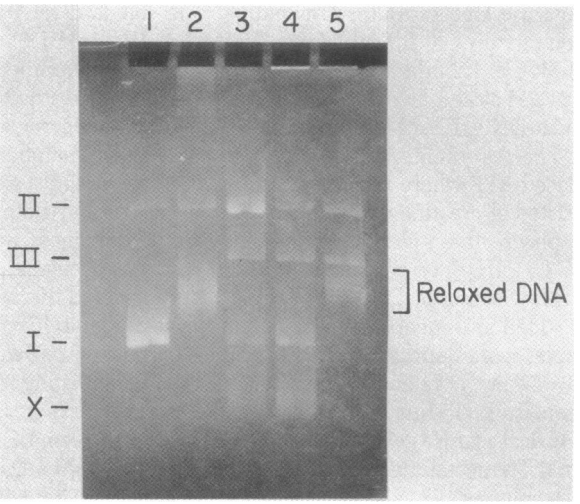


FIG. 3. Relaxation of form I and form X DNA by *E. coli* topoisomerase I. Lanes: 1, form I DNA; 2, product made by treatment of form I DNA with topoisomerase I; 3, standard reaction as diagrammed in Fig. 1, yielding form I and form X; 4, standard reaction, except that EDTA, $NaDodSO_4$, and proteinase K were omitted and instead the sample was heated to 65°C for 5 min to inactivate recA protein and ligase after the reaction; 5, same as lane 4, then treated with topoisomerase I.

Requirements for Unwinding. Unwinding of DNA by recA protein was undetectable when ATP or an ATP-regenerating system was omitted (data not shown) or when single-stranded

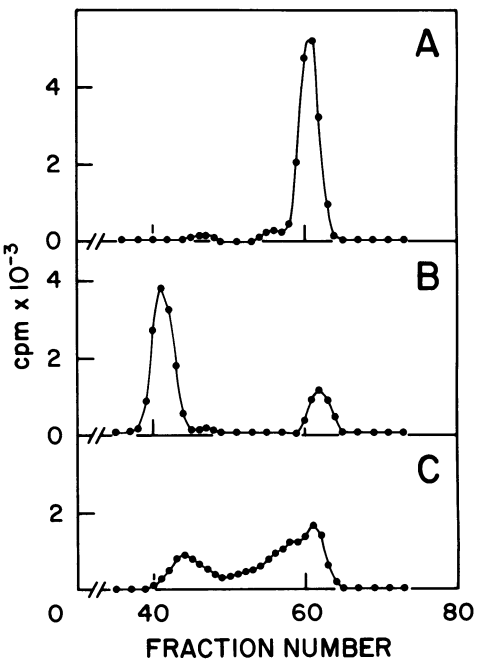


FIG. 4. Decreased linking number of circular DNA sealed in the presence of recA protein and homologous single-stranded DNA. M13G*ori*1 [^{3}H]DNA nicked by *Xho* I was allowed to react with M13 single-stranded circular DNA for 25 min at 37°C in the presence of creatine phosphokinase (50 units/ml) and 2.3 mM phosphocreatine. After isopycnic banding in CsCl/propidium diiodide, gradients were collected from the bottom of the tube. (*A*) DNA ligase was omitted. The large peak contains form II and form III DNA; a small amount of form I DNA was also present in the starting material. (*B*) recA protein was omitted. The large peak at the bottom of the gradient contains molecules that were relaxed at the time of ligation. The small peak at the starting position consists of forms II and III DNA that was not sealed. (*C*) The products formed in the complete reaction, as diagrammed in Fig. 1.

DNA was omitted (Fig. 2, lane 1). In the presence of ATP, extensive unwinding, as revealed by the production of the form I band and the X band, required a high degree of homology of the single-stranded and double-stranded DNA. Single-stranded ϕX174 DNA did not serve as a cofactor for unwinding G4 DNA, which is 70% homologous (Fig. 5). However, as expected from previous results (16, 17), ϕX174 single-stranded DNA did promote the unwinding of G4 DNA in the presence of the nonhydrolyzable analog ATPγS (Fig. 5, lane 7), an observation that provides a positive control for this experiment.

Time Course of Unwinding and Inhibition of Unwinding by ADP. We studied the time course of appearance of DNA in the form I band and in the X band by cutting these bands out of the gels and measuring the radioactive DNA in them. DNA in the form I band reached its maximum concentration within 20 min and subsequently decreased (Fig. 6*B*); the concentration of DNA in the X band increased more slowly and reached its maximum later (Fig. 6*A*). This pattern is consistent with a precursor–product relationship in which the less unwound DNA is an intermediate in the production of the more unwound DNA. The time course of appearance of DNA in the form I band is the same as the time course of formation of paranemic joint molecules made from similar substrates (unpublished data).

To relate unwinding and synapsis further, we decreased the concentration of phosphocreatine, which results in the earlier accumulation of ADP and consequent inhibition of synapsis (9). This caused an earlier decrease in the amount of DNA found in the form I band (Fig. 6*B*), and a lower yield of the more unwound DNA in the X band (Fig. 6*A*).

Association of Single-Stranded DNA with the Unwound DNA in a Synaptic Structure. The unwinding observed in the experiments described above requires synapsis and a high degree of homology of the paired but noninterwound strands. The association of the single strand with the unwound helix was revealed by further observations on the joint action of *E. coli* topoisomerase I and recA protein. Previous studies have shown that recA protein can make paranemic joint molecules from circular single strands and homologous superhelical DNA and that *E. coli* topoisomerase I can interwind the circular single strand with its complement, thereby creating a topologically linked structure that we called a "hemicatenane" (24). Using an ATP-regenerating system to promote synapsis involving nonsuperhelical DNA, we did the same experiment with M13G*ori*1 duplex DNA cleaved at the *Xho* I site in the G4 region to produce linear molecules. Topoisomerase I stabilized joint molecules made by M13 circular single strands but, as expected, had no effect on the stability of joint molecules made by G4 circular single strands (Table 2). Experiments that will be reported elsewhere have shown that this stabilization is attributable to topological interwinding of the M13 circular single strand with its complement.

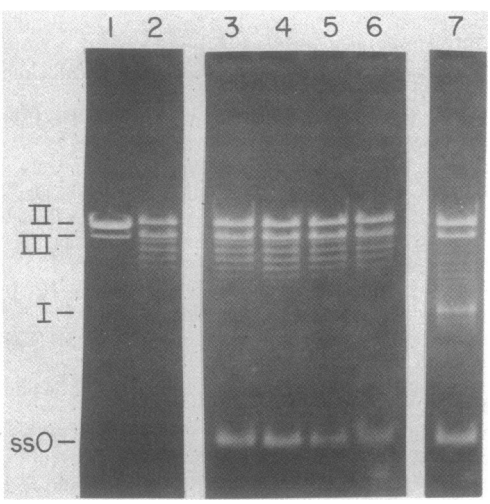


FIG. 5. Failure of ϕX174 single-stranded DNA to promote extensive unwinding of G4 duplex DNA. Lanes: 1, starting material (forms II and III G4 DNA made by cleavage with *Xho* I); 2, reaction in absence of single-stranded DNA cofactor (the ladder of bands is a set of topological isomers of DNA that was relaxed at the time of closure; the set migrates as slightly negatively superhelical DNA in these gels); 3, complete reaction with ϕX174 single-stranded circular DNA as cofactor without ATP regenerating system; 4–6, as lane 3 but with creatine phosphokinase (50 units/ml) and 1.5 mM phosphocreatine [incubation was at 37°C for 10 min (lane 4), 20 min (lane 5), or 30 min (lanes 2, 3, 6, and 7)]; 7, positive control, standard reaction with ATPγS instead of ATP.

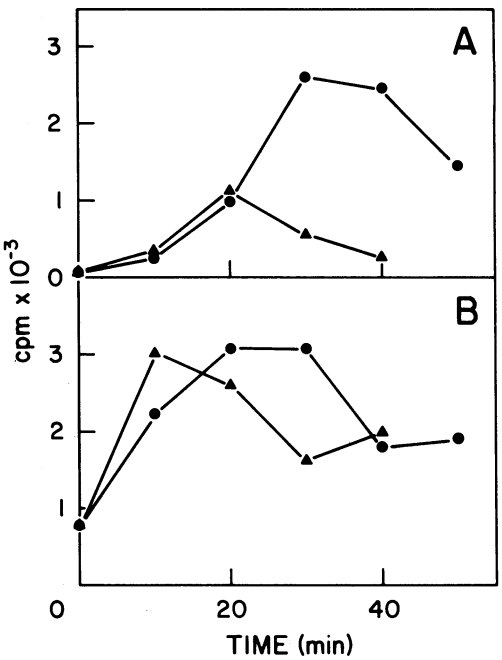
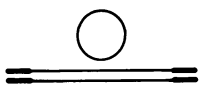


FIG. 6. Time course of formation of unwound DNA and inhibitory effect of accumulating ADP. The time course of appearance of form I DNA (*B*) and the X band (*A*) DNA were measured in the presence of an ATP-regenerating system with two different concentrations of phosphocreatine. The reaction mixtures contained 24 μM M13G*ori*1 [^{3}H]DNA nicked by *Xho* I and 12 μM M13 single-stranded circular DNA and were incubated at 37°C for the indicated times. The form I band and the X band were cut out of the gels and their radioactivity was measured as described. ▲, 1.2 mM phosphocreatine; ●, 2.4 mM phosphocreatine.

DISCUSSION

These experiments show that pairing of nonsuperhelical DNA with homologous single-stranded DNA is accompanied by extensive unwinding of the duplex DNA under conditions in which the formation of an interwound or plectonemic joint is topologically blocked by flanking heterologous sequences. Thus, in such a case, homologous pairing must be mediated by a paranemic joint in which the newly paired strands are not interwound. Earlier indirect evidence (7) and more recent direct observations (unpublished data) have shown that paranemic joints are less stable than plectonemic joints, as one might expect. Consequently, when the formation of a plectonemic joint is blocked, the steady-state concentration of joint molecules, and hence of unwound duplex DNA, depends upon the rate of dissociation of the unstable paranemic joint relative to the rate of reassociation or synapsis. The sensitivity of synapsis to inhibition by ADP (9) explains why unwinding by recA protein in the presence of ATP requires an ATP-regenerating system.

Table 2. Action of *E. coli* topoisomerase I on a paranemic joint in linear duplex DNA



Circular single strands	Topoisomerase I	D loops, % Unheated	D loops, % Heated, 80°C, 4 min
M13	−	47	5
	+	75	68
G4	−	75	9
	+	85	10
ϕX	+	2	—

Joint molecules were formed with duplex M13G*ori*1 DNA cleaved at the *Xho* I restriction site and circular single-stranded M13 DNA. The reaction mixture (50 μl) contained 3.3 μM single-stranded DNA, 2 μM double-stranded DNA, 31 mM Tris·HCl (pH 7.5), 12.5 mM $MgCl_2$, 1.25 mM ATP, 3 mM phosphocreatine, 5 units of phosphocreatine kinase per ml, 3.5 mM dithiothreitol, 88 μg of bovine serum albumin per ml, 3.3 μM recA protein, and 4% (vol/vol) glycerol; incubation was at 37°C. After 20 min, 11 nM *E. coli* topoisomerase I or an equal volume (1 μl) of recA buffer was added and the incubation was continued for 5 more min. The reaction mixture was then diluted with 450 μl of 1.5 M NaCl/0.15 Na citrate and kept on ice. Aliquots were either diluted 1:120 in cold 1.5 M NaCl/0.15 M Na citrate and filtered through nitrocellulose or diluted 1:20 in NaCl/Na citrate at 80°C, incubated for 4 min, diluted 1:6 with cold NaCl/Na citrate, and filtered.

An unwinding activity of recA protein has been described in two other systems. Ohtani *et al.* (21) recently reported that, in the presence of ATP and homologous fragments of single-stranded DNA, recA protein can extensively unwind superhelical DNA, even to the extent of producing positively superhelical domains. In this case, after introduction of recA protein to the duplex molecule at the site of a D loop, the recA protein progressively invaded and unwound the duplex molecule. Our results show that neither formation of a true D loop nor superhelicity is required for this helicase activity.

Elegant electron microscopic observations made in Koller's laboratory (18, 19) have revealed that, in the presence of ATPγS, recA protein forms a regular filament with duplex DNA in which the latter is partially unwound. This structure appears to reflect the properties that enable recA protein, in the presence of ATP, to unwind duplex DNA extensively and to form paranemic joints. However, as demonstrated in this report, extensive unwinding of duplex DNA in the presence of ATP requires single-stranded DNA that has a high degree of homology, which indicates that under normal conditions the single-stranded DNA is instrumental in the unwinding. By contrast, in the presence of ATPγS, extensive unwinding of duplex DNA can occur without any added single-stranded DNA, at least under some conditions (18, 19), and can be stimulated by heterologous DNA or even oligodeoxyribonucleotides (16, 17, 22). The experiments reported here do not exclude the possibility that, in the presence of ATP, unwinding of a few turns of DNA might be stimulated by heterologous or partially homologous DNA. Indeed, other experiments have suggested indirectly that G4 single-stranded DNA stimulates an initial unwinding of superhelical ϕX174 DNA, which is 70% homologous (31).

Under normal conditions in the presence of ATP, single-stranded DNA introduces recA protein to duplex DNA and creates the synaptic structure whose features we have begun to explore. An interesting property of the paranemic joint—the DNA part of the synaptic structure—is its suitability as a substrate for *E. coli* topoisomerase I which is capable of changing the interwinding of complementary strands only when they have a deficit of turns about one another (29, 30). That property leads us to suppose that all three strands in the paranemic joint are underwound relative to duplex DNA at the same ionic strength. We previously proposed that recA protein forms a nascent heteroduplex structure in which the protein holds the strands of the duplex DNA in an unwound configuration and provisionally pairs a third strand with its complement (9). The present observations support that view: they show that, under fully functional conditions, recA protein can hold 1,000 or more base pairs in an unwound configuration, part or all of which is a paranemic joint.

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